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Recovery of genetic diversity in threatened plants through use of germinated seeds from herbarium specimens

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Abstract

The reintroduction of *ex situ* conserved individuals is an important approach for conserving threatened plants and reducing extinction risk. In this study, we elucidated the effects on the genetic diversity of wild populations of *Vincetoxicum pycnostelma* Kitag. [= *Cynanchum paniculatum* (Bunge) Kitag.] by modelling the genetic consequences of reintroducing plants using the germinated seeds of herbarium specimens. This semi-natural grassland herb is threatened in Japan. First, we tested the germinability of seeds from herbarium specimens collected from nine sites in Kinki and Tokai districts, Japan (one specimen per site, total 206 seeds). Next, we analysed the genetic diversity and structure of germinated seedlings and the current wild individuals using nine polymorphic microsatellite markers. Germination was observed for 38 seeds (18.4%) from four specimens collected 3–18 years prior to the study. Although the genetic diversity of the specimens' seeds was lower than that of the wild population because of the small sample size, the seedlings from specimens taken from three sites had unique alleles that did not exist in the wild populations. Consequently, viable herbarium specimens' seeds with unique alleles could be useful resources for recovering the genetic diversity in threatened wild plant populations.

Keyword: threatened plants, genetic diversity recovery, germination test, microsatellite, reintroduction

Introduction

Many species worldwide face an extinction crisis because of the destruction and fragmentation of habitats as a result of human activities (Ceballos and Ehrlich 2002; Hooper et al. 2005; Aguilar et al. 2006). To conserve the populations of threatened species, it is crucial to maintain not only population size and habitat but also genetic diversity for preventing the decline in reproductive success owing to inbreeding depression, which would escalate the extinction risk (Keller and Waller 2002; Mattila et al. 2012; Palomares et al. 2012). Furthermore, a loss of genetic diversity would compromise their adaptive potential, particularly in the face of concerns pertaining to climate change (Blows and Hoffmann 2005; Willi et al. 2006).

Although the conservation of natural habitat is critical for conserving ecological interactions, *in situ* and *ex situ* conservation is also appropriate for safeguarding individual species against extinction in the wild. For example, use of soil seed bank is an *in situ* natural resource for the reproduction of plants that is effective for the restoration of genetic diversity (Uesugi et al. 2007; Zaghloul et al. 2013). On the other hand, the primary purpose of *ex situ* conservation is to maintain wild species outside their natural habitat so that species recovery and reintroduction can be attempted if the wild populations severely decline or become extinct. It is well known that preserved seeds are useful as *ex situ* populations (Honnay et al. 2008; Frankham et al. 2009; Hoban and Schlarbaum 2014). The reintroduction of many individuals germinated from preserved seeds also allows the recovery of population size and genetic diversity (Honnay et al. 2008; Guerrant et al. 2014; Hoban and Schlarbaum 2014). Thus, projects to preserve the seeds of many plants are used worldwide and comprise an efficient method for plant reintroduction and conservation (Schoen and Brown 2001; Guerrant et al. 2014; Hoban and Schlarbaum 2014). For example, the ‘Millennium

Seed Bank Project' of the Royal Botanic Gardens, Kew, the 'Svalbard Global Seed Vault' and many other botanical gardens store wild and cultivated plant seeds as a resource for future use in conservation translocations and crop development (Schoen and Brown 2001; Van Slageren 2003; Qvenild 2008; Alsos et al. 2013).

However, re-establishment of the seeds collected in the past has risks pertaining to inbreeding and inbreeding depression if the sources are restricted or population size is small (Frankel and Soulé 1981; Schoen and Brown 2001). In addition, reintroduced individuals collected from remote habitats would also have reduced fitness because of local adaptation (Becker et al. 2006; Leimu and Fischer 2008; Hereford 2009). Furthermore, reintroduced individuals that are genetically distinct from a wild population would not only have a risk of outbreeding depression (Montalvo and Ellstrand 2001; Huff et al. 2011), but would also lead to the loss of the local genetic identity of the native population (Gottelli et al. 1994; Milián-García et al. 2014). Accordingly, to conserve threatened plants by reintroducing individuals from preserved seeds, it is very important to collect many seeds from widely dispersed populations (Falk and Holsinger 1991; Hoban and Schlarbaum 2014). However, collecting seed resources of threatened or locally extinct species from wild populations may be difficult, although seed banking projects throughout the world have been recently constructed and followed the protocols to ensure the maintenance of genetic diversity (León-Lobos et al. 2012; Guerrant et al. 2014; Hoban and Schlarbaum, 2014). Herbarium specimens in museums can retain viable and germinable diaspores (Windham et al. 1986; Bowles et al. 1993; Lledó et al. 1996; Magrini et al. 2010; Magrini 2011; Bewley et al. 2013; Shiga 2013); therefore, they have potential as a reintroduction resource. Furthermore, the viable diaspores in herbaria specimens are also useful for the conservation of threatened local populations in each plant species because presumably the

specimens are collected from these populations. Despite the potential use of herbarium specimens' seeds, the effect of reintroduced seeds on the restoration of genetic diversity has not been studied, and it is unknown whether genetic diversity now lost in wild populations can be found in herbarium collections.

Vincetoxicum pycnostelma Kitag. [= *Cynanchum paniculatum* (Bunge) Kitag.] (Fig. 1a), subfamily Asclepiadoideae of Apocynaceae, is a perennial herb that grows in semi-natural grasslands in Japan, Korea, China, Mongolia and Russia (Yamazaki 1993; Wu and Raven 1995; Flora of Korea Editorial Committee 2007). Although this species was common a few decades ago, its populations have rapidly declined because of land-use changes in semi-natural grasslands in Japan (Environment Agency of Japan 2000; Uematsu et al. 2010). It is estimated that the extinction probability after 100 years is 96% (Environment Agency of Japan 2000). The species is categorized as 'near threatened' in the Japanese Red List (Ministry of the Environment Government of Japan 2012). Many other native herbaceous plants have also experienced a rapid decline in semi-natural grasslands (Koyanagi and Furukawa 2013); hence *V. pycnostelma* is a prime example of these rare and threatened species.

In this study, we examined the germinability of seeds from herbarium specimens of *V. pycnostelma* and we assessed the effect on the genetic diversity and structure of the wild populations by modelling the reintroduction of germinated seeds. We also discuss suitable methods for collecting and managing herbarium specimens' seeds at museums that are to be used for conserving not only threatened plants but also the local populations in each species.

Materials and methods

Selection of herbarium specimens

Seeds were collected from the herbarium of the Osaka Museum of Natural History, Japan (Figs. 1a, b). The museum was established in 1974, and the herbarium preserves more than 270,000 vascular plant specimens (Osaka Museum of Natural History 2012). In this herbarium, insect control involved naphthalene application and fumigation with carbon disulphide; the room temperature was not controlled until 2000. However, since 2001, integrated insect control has been performed by applying naphthalene and freezing treatments and the room conditions have been controlled at 20°C and 50% humidity. In the herbarium, 206 seeds from nine specimens with mature fruits (one specimen per site) from Kinki and Tokai districts, Japan, had been collected (Fig. 2). The ages of the nine stored specimens were from 4 to 62 years (Table 1). Seeds were collected from only one fruit per specimen. After the germination test, specimens of the seedlings or seeds were mounted and donated to the Osaka Museum of Natural History with annotation cards.

Germination test of herbarium specimens' seeds

Germination of the herbarium specimens' seeds was tested using the screening test system (Washitani 1987) under a 12/12-h photoperiod and two temperature regimes: 1) a gradually increasing temperature regime from 4°C to 36°C at intervals of 4°C (IT) and 2) a gradually decreasing temperature regime from 36°C to 4°C at intervals of 4°C (DT). The two treatments were conducted because the preservation condition in the herbarium may have affected the dormancy of the specimens' seeds, and it was predicted that a cold stratification treatment would be required to break the seed dormancy of *V. pycnotelma* (Zhou et al. 2003). Considering that growth rate increases with temperature, the duration of the higher temperature treatment was less than the duration of the lower temperature treatment (Table S1). After reaching the final temperature in each regime, these

seeds were incubated at 25°C (DT, 5 days) or treated at alternating temperatures of 12°C and 25°C at 24-h intervals (IT, total 5 days). The number of germinated seedlings was counted when the temperatures were changed, and the seedlings were moved to an incubator at 25°C to determine whether they would grow normally. For microsatellite analysis, we selected well-growing plants among 32 samples of 38 seedlings obtained from four specimen sheets. The germination test was conducted in June 2012 (Site i) and May 2013 (Sites a–h; Table 1).

The ungerminated seeds were tested using tetrazolium (2,3,5-triphenyl tetrazolium chloride) to examine their viability (Cottrell 1947; Elias et al. 2012). The seeds were cut and stained for 48 h in the dark with 1% tetrazolium solution, and seeds that stained red were defined as viable.

Sampling and microsatellite analysis

In 2012 and 2013, leaf samples from 131 adult individuals were collected from sites same as those from where the herbarium specimens had been collected (Figs. 1c and 2). The samples were used to estimate the genetic diversity among the extant populations (Table 2). At all sites, we comprehensively collected samples from each entire patch. Because *V. pycnotelma* at Site e was locally extinct, we collected leaf samples from a neighbouring population located one kilometre away. The number of individuals was counted at each site.

Genomic DNA was extracted using a modified cetyltrimethylammonium bromide method (Milligan 1992). The genotypes of each individual, including wild populations and specimens' seedlings, were characterized at nine microsatellite loci. Seven of the nine loci were characterized by Nakahama et al. (2012): *Vpy002*, *Vpy006*, *Vpy012*, *Vpy013*, *Vpy16*, *Vpy018* and *Vpy022*. Two of the nine loci were developed by Nakahama et al. (unpublished data): *Vpy025* (GenBank accession

number: AB948217) and *Vpy031* (GenBank accession number: AB948218). Polymerase chain reaction (PCR) amplifications, except for those of *Vpy025* and *Vpy031*, were performed following the standard protocol of the Qiagen Multiplex PCR kit (Qiagen) with a final reaction volume of 5 μL, which contained 16 ng extracted DNA, 2.5 μL of 2× Multiplex Master Mix and 0.2 μM of each multiplexed primer. For *Vpy025* and *Vpy031*, the forward primer was synthesized with the M13 tag sequence (*Vpy025* 5′-CACGACGTTGTAAAACGAC-3′, *Vpy031* 5′-TGTGGAATTGTGAGCGG-3′; Boutin–Ganache et al. 2001). The PCR mixtures of *Vpy025* and *Vpy031* had a final volume of 5 μL, which included 16 ng extracted DNA, 2.5 μL of Multiplex PCR Master Mix, 0.01 μM forward primer, 0.2 μM reverse primer and 0.1 μM M13 fluorescent primer. The PCR amplifications of all loci were carried out using a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Tokyo, Japan) using the following conditions: initial denaturation at 95°C for 15 min, followed by 25 cycles of 30 s at 94°C, 1.5 min at 57°C and 1 min at 72°C, and a final extension for 30 min at 60°C. The PCR product size was measured using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) and GeneMapper ver. 4.1 (Applied Biosystems).

Statistical analysis of the genetic diversity and structure

Genetic diversity was evaluated for the specimens' seedlings, the wild populations and the hypothetical mixed populations, which comprised a hypothetical mixing of the specimens' seedlings and each corresponding wild population. The genetic diversity was evaluated in terms of the following: average number of alleles per locus, allelic richness (El Mousadik and Petit 1996), summed number of rare alleles with frequencies less than 5% among the total population, expected heterozygosity, observed heterozygosity and inbreeding coefficient. We also evaluated the summed

number of unique alleles that were only present in specimens' seedlings from each site. All of these parameters, except allelic richness summed number of rare and unique alleles, were calculated using GenAlEx version 6.41 (Peakall and Smouse 2006). Allelic richness, deviation from Hardy–Weinberg equilibrium and the linkage disequilibrium between loci were also determined using FSTAT ver. 2.9.3 software (Goudet 2001). The significances of the heterozygosity excess and deficit values were tested by comparison with the 95% confidence intervals derived from 108,000 randomizations. We evaluated the genetic relationships between the wild populations at all sites using Bayesian clustering with STRUCTURE ver. 2.3.4. (Pritchard et al. 2010), which assigns individuals into *K* clusters. The population structure was simulated with the values of $K = 1–8$ under an admixture model and the correlated allele frequency model (Hubisz et al. 2009). All runs involved one million Markov chain Monte Carlo iterations after a burn-in period of one million iterations. Twenty runs were performed for each value of *K*. The *F* value, the estimated amount of genetic drift between each cluster and a common ancestral population and the expected heterozygosity were calculated. The number of clusters was determined by comparing the mean values and the variability of log likelihoods for each run. To select the optimal value of *K*, STRUCTURE HARVESTER was used (Earl and vonHoldt 2012). We also evaluated genetic relationships between the wild populations and specimens' seedlings at each site by the same method.

To evaluate genetic differentiation between specimens' seedlings and wild populations for each site at the individual level, we calculated pairwise co-dominant genotypic distances (Smouse and Peakall 1999) between all specimens' seedlings and wild individuals from all sites. We also performed principal co-ordinates analysis (PCoA) using GenAlEx version 6.41 (Peakall and Smouse 2006).

196

197 **Results**

198 Germination and viability of herbarium specimens' seeds

199 A total of 38 seeds, representing four herbarium specimens, were germinated out of a total of 206
200 seeds (Fig. 1d and Table 1). The germination percentage for seeds from each specimen ranged from
201 0% to 56.5%, with an average of 18.4%. The oldest germinated specimens' seeds were collected 18
202 years prior to the study. Under the IT and DT conditions, seed germination from two and four
203 specimens were observed, respectively. Each 18.4% of the seeds germinated in both conditions.
204 Furthermore, 3.0% of ungerminated seeds were confirmed to be viable according to the tetrazolium
205 dye test (Table 1). The viable seeds were collected from three herbarium specimens, which were
206 collected 9 to 18 years prior to the study. The ungerminated viable seeds were confirmed only in the
207 IT condition.

208

209 Genetic diversity of seedlings from herbarium specimens, wild populations and hypothetical mixed
210 populations

211 There was no evidence for large allele dropouts or null alleles in the data set. The numbers of alleles,
212 the allelic richness and the expected heterozygosity of herbarium specimens' seedlings was
213 considerably lower than those of the wild populations because their allele frequencies were
214 dominated by a few alleles, although the observed heterozygosity of the seedlings was similar
215 between wild populations and specimens seedlings at all sites. At Sites e, g and i, the number of
216 alleles of hypothetical mixed populations were higher than those of the wild populations because one
217 to three unique alleles existed only in the seedlings. The allelic richness of the hypothetical mixed

218 populations was higher than that of the wild populations only at Site e. Although rare alleles were
219 much more common in the wild populations than in the seedlings at all sites, the seedlings had some
220 rare alleles at all sites. Unique alleles in the specimens' seedlings were found at the three sites except
221 Site d. At Sites e and i, the expected heterozygosity of the hypothetical mixed populations was more
222 than that of the wild populations. The inbreeding coefficient of the seedlings ranged from −0.767 to
223 −0.205, whereas that of the seedlings at Site i indicated significant heterozygosity excess (Table 2).
224 On the other hand, no wild populations exhibited significant heterozygosity excesses. Significant
225 linkage disequilibrium occurred at Site g for only one pair of loci (*Vpy002*/*Vpy013*; $P < 0.05$).
226
227 Genetic differentiation and structure of herbarium specimens' seedlings and wild populations
228 The STRUCTURE analysis indicates that the wild populations were divided into distinct genetic
229 clusters (Fig. 3a). The ΔK value representing the hierarchical approach for the STRUCTURE
230 analysis was clearly the highest at $K = 2$ (Fig. S1a). In addition, the variance of log likelihood
231 between runs was low and the ΔK value was high at $K = 3$ (Fig. S1a). Therefore, $K = 3$ also yielded
232 meaningful results. Thus, the results obtained with $K = 2$ and $K = 3$ are shown (Fig. 3a). When $K = 2$,
233 the individuals were divided into two clusters. The wild populations at Sites d, e and i were assigned
234 to cluster I, and the wild population at Site g was assigned to cluster II. The *F* value of cluster I was
235 higher than that of cluster II, and the expected heterozygosity of clusters I and II were 0.780 and
236 0.747, respectively (Fig. 3a). When $K = 3$, cluster I from the $K = 2$ analysis was divided into two
237 clusters. The wild population at Site g was assigned to cluster II and those at Sites e and i were
238 assigned to cluster III. The *F* value of cluster III was lower than that of cluster I and II, and the
239 expected heterozygosity of cluster I, II and III were 0.743, 0.742 and 0.781, respectively (Fig. 3a).

The wild populations and specimens' seedlings were also divided into distinct genetic clusters (Fig. 3b). The ΔK value representing the hierarchical approach for the STRUCTURE analysis was clearly highest at $K = 3$ (Fig. S1b). Thus, $K = 3$ was the uppermost hierarchical level of the genetic structure. In addition, the variance of log likelihood between runs was low and the ΔK value was high at $K = 4$ (Fig. S1b). Therefore, $K = 4$ also yielded meaningful results. Thus, the results obtained with $K = 3$ and $K = 4$ are shown (Fig. 3b). When $K = 3$, the individuals were clearly divided into three clusters. The wild population and herbarium specimens' seedlings at Site d and the seedlings at Site e were assigned to cluster I. The wild populations at Sites e, g and i were assigned to cluster II. The seedlings at Sites g and i were assigned to cluster III. The F values of cluster I and III were higher than that of cluster II, and the expected heterozygosity of clusters I, II and III were 0.672, 0.787 and 0.584, respectively (Fig. 3b). When $K = 4$, cluster II from the $K = 3$ analysis was divided into two clusters. The wild population at Site g was assigned to cluster II and the wild populations at Sites e and i were assigned to cluster III. The F values of cluster I and IV were higher than those of cluster II and III, and the expected heterozygosity of cluster I, II, III and IV were 0.667, 0.744, 0.786 and 0.584, respectively (Fig. 3b).

According to PCoA based on co-dominant genotypic distances, about 48.9% of the total variation was described by the first two axes (Fig. 4). The specimens' seedlings and the wild individuals were plotted as a single group at Site d. On the other hand, the plots of specimens' seedlings at Sites e and g were nearby the wild individuals. The plots of seedlings at Site i were markedly removed from those of the wild individuals.

Discussion

We found that 18.4% of specimens' seeds were germinated. Furthermore, the specimens' seedlings had unique alleles that did not exist in the wild populations, although the genetic diversity of germinated specimens' seedlings was lower than that of wild populations and clear genetic structure was observed between specimens' seedlings and wild populations at the three sites. These result suggested that viable herbarium-specimen sourced seeds can contribute to the restoration of genetic diversity in threatened plants as *ex situ* conservation resources.

Restoration of genetic diversity by specimens' seedlings

The specimens' seedlings from each site had one to three unique alleles (Table 2). Although we could not estimate the past genetic diversity in each site, the number of alleles of *V. pycnostelma* may have declined in recent decades. The specimens' seedlings probably had the alleles that had extirpated from current wild populations. Thus, the reintroduction of specimens' seedlings with these unique alleles would be useful to restore or augment the genetic diversity of the wild populations.

The genetic diversity (i.e. number of alleles, allelic richness and expected heterozygosity) of specimens' seedlings was lower than that of the wild population. In this study, we used only one specimen fruit per population in order to minimize the damage to the specimens. Because the subfamily Asclepiadoideae of Apocynaceae is pollinated via transfer of pollinia, multiple paternities are very low within fruits (Broyles and Wyatt 1990; Wyatt and Broyles 1994). Therefore, the genetic diversity of the specimens' seedlings would be enhanced by using the seeds of (1) multiple fruits and specimens, (2) species that do not form pollinia (i.e. Orchidaceae and Asclepiadoideae) and allogamous species and (3) fruits from chasmogamous flowers.

Except at Site d, the allele compositions were different and clear genetic structures were

observed between the specimens' seedlings and the wild populations (Figs. 3 and 4). This could be because the specimens' seedlings retained different alleles than the wild populations and they exhibited low genetic diversity. Similarly, significant genetic differences between the seedlings derived from soil seedbanks and the wild populations were also reported (Honnay et al. 2008). Reintroduction of many seedlings taken from only a few specimens or fruits might lead to the dominance of a few alleles in the wild populations. Thus, collecting seeds from multiple fruits and specimens would enhance the genetic diversity of specimens' seedlings so that they more closely resemble the original populations, and the allele frequencies of the seedlings would then be more similar to the wild populations.

Usability of herbarium specimens' seeds of threatened plants

In this study, we determined that 18.4 % of specimens' seeds, which had been collected recently (<19 years), were germinated. These germinated seeds preserved in herbarium could be useful in reintroduction or augmentation resources because viable seeds and bulbils of plants can be preserved for a long time (>20 years) under ideal conditions (Probert et al. 2009; Alsos et al. 2013). In addition, seeds that possess physical dormancy and those with large embryos and little endosperm remain viable for a longer time (Merritt et al. 2014). The seeds of *V. pycnostelma* also possess a physiological dormancy and *Vincetoxicum* species have large embryos (Martin 1946; Zhou et al. 2003; Baskin and Baskin 2014). Thus, the seeds of *V. pycnostelma* specimens collected more than 20 years prior to the study would have a potential to germinate, although we used only nine fruits for germination test in this study. Furthermore, 3.0% of the ungerminated specimens' seeds that were collected 9–18 years prior to the study were viable (Table 1). Because such seeds may be in a

306 dormant state, seed dormancy should be broken using various methods such as cold stratification,
307 chemicals, heat shock, hormones or scarification (Fontaine et al. 1994; Susko et al. 2001; Kanmegne
308 and Omokolo 2008; Baskin and Baskin 2014). To germinate seeds, it is important to create
309 appropriate environments for inducing germination based on the germination characteristics of the
310 target species. Zygotic embryo culture and callus culture technologies are also available for
311 regenerating endangered plants (Gomes et al. 2003; Rambabu et al. 2006). These technologies could
312 be used to increase the number of individuals derived from the specimens' seeds. The storage
313 conditions in herbaria are also important for specimens' seed longevity. Insect controls that avoid
314 heating, but employ nitrogen, argon, carbon dioxide and freezing have been developed in herbaria
315 (Strang 1992; Valentin 1993); these controls do not have negative effects on seed germination of
316 many species (Bass and Stanwood 1978; Prokopiev et al. 2014).

317 It is recommended that sampling regimes for reintroduction resources should involve collecting 1
318 to 20 seeds per individual from each of 10 to 50 individuals belonging to each of five separate
319 populations to remove the inbreeding depression (Falk and Holsinger 1991). Hoban and Schlarbaum
320 (2014) also suggest that we should collect seeds from 25 to 30 individuals belonging to the few but
321 widely dispersed populations and between two to eight seeds of fruits per individual to maintain
322 their genetic diversity. However, many specimens of target species collected from the same site (>10
323 individuals) may be rarely preserved in a herbarium. In addition, damage to an excessive number of
324 specimens should be minimized because museum specimens are very valuable and irreplaceable
325 (Graves and Braun 1992; Wandeler et al. 2007; Shiga 2013). Furthermore, the number of retained
326 and available seeds on herbarium specimens would be also not known before their germination tests.
327 Thus, it may be difficult to collect sufficient seeds from specimens in only a few herbaria as

reintroduction resources. For example, it is feasible to sample across many sites and have wider genetic variation if material is sourced from several herbaria.

In recent decades, in addition to the designation of the sampling guidelines of seed banking projects, their utilization for *ex situ* conservation has been increasingly applied to a more diverse array of wild species (Schoen and Brown 2001; Van Slageren 2003; Guerrant et al. 2014; Hoban and Schlarbaum 2014). However, the application of seed banking projects may be difficult for the conservation of locally threatened populations because these projects usually do not assume the conservation of the local populations. The use of herbaria specimens' seeds would also remarkably contribute to restore the genetic diversity of not only the plant species but also each locally threatened population if the viable specimens' seeds collected at the target species or populations are preserved in herbaria.

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526 **Supplementary material**

527 Additional supplemental material can be found in the online version of this article.

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529 **Table S1** The condition of germination test

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531 **Figure S1** The ΔK in the STRUCTRE analysis

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548 **Table 1** Specimen characteristics and number of seeds used in experiments

Site	Prefecture	No. of years after collected	Date of collecting specimens	Voucher specimen	Increasing temperature condition			decreasing temperature condition		
					No. of seeds used experiment	No. of germinated seeds	No. of viable ungerminated seeds	No. of seeds used experiment	No. of germinated seeds	No. of viable ungerminated seeds
a	Gifu	16	25 Sep. 1997	H. Marui 2434 (OSA203391)	10	0	1	10	0	0
b	Kyoto	51	16 Sep. 1962	M. Hutoh 24248 (OSA214994)	9	0	0	9	0	0
c	Kyoto	22	24 Sep. 1991	T. Fujii 2413 (OSA209484)	10	0	0	10	0	0
d	Nara	4	12 Oct. 2009	S. Onoue s.n. (OSA225354)	9	3	0	9	3	0
e	Nara	16	03 Aug. 1997	K. Seto 47673 (OSA104247)	11	0	0	11	4	0
f	Nara	62	03 Jul. 1951	M. Hori s.n. (OSA190777)	8	0	0	8	0	0
g	Osaka	3	26 Nov. 2010	F. Uwakubo 101126-002 (OSA185864)	12	0	0	12	2	0
h	Osaka	9	10 Oct. 2004	K. Hirano 2501 (OSA280257)	11	0	2	11	0	0
i	Hyogo	18	02 Nov. 1994	S. Miyake 2987 (OSA122127)	23	16	2	23	10	0
				Total	103	19	5	103	19	0

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Table 2 Genetic diversity measurements of each site of *Vincetoxicum pycnostelma*. Each site contained specimens' seedlings, a wild and a hypothetical mixed population (specimens' seedlings + wild population). *A*, numbers of alleles per locus; *AR*, allelic richness; *RA*, summed number of rare alleles; *UA*, summed number of unique alleles that are only present in specimens' seedlings; H_O, observed heterozygosity (in bold numbers, if values are significantly deviated from HWE); H_E, expected heterozygosity; F_{IS}, inbreeding coefficient

Site	Wild Population size	Sample	No. of samples	*A*	*AR*	*RA*	*UA*	H_O	H_E	F_{IS}
d	20	specimens' seedlings	6	3.111	2.366	5	0	0.815	0.582	-0.382
		Wild population	19	6.222	2.683	17		0.754	0.687	-0.106
		Hypothetical mixed population	25	6.222	2.637	17		0.769	0.680	-0.137
e	220*	specimens' seedlings	4	3.000	2.406	5	1	0.778	0.566	-0.348
		Wild population	46*	11.333	2.917	51		0.749	0.752	0.008
		Hypothetical mixed population	50	11.444	2.929	52		0.751	0.756	0.010
g	100	specimens' seedlings	2	2.222	2.222	2	1	0.833	0.472	-0.767
		Wild population	50	10.000	2.859	39		0.767	0.748	-0.030
		Hypothetical mixed population	52	10.111	2.858	40		0.769	0.748	-0.034
i	24	specimens' seedlings	20	2.889	2.230	3	3	**0.694**	0.570	-0.205
		Wild population	16	7.556	2.826	25		0.701	0.723	0.030
		Hypothetical mixed population	36	8.000	2.765	27		0.698	0.725	0.044

*: sampled about 1 km from where specimens were originally collected, because original population was locally extinct

557 **Figure captions**

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559 **Fig. 1 a** The herbarium specimen of *Vincetoxicum pycnostelma* collected site i (OSA122127). Arrow head
560 indicates the fruit, Bar indicates 50mm. **b** Seeds in the fruits of OSA122127. Bar indicates 5.0mm. **c**
561 Habitat of *V. pycnostelma* (Site d). **d** Juvenile individuals germinated from the seeds of herbarium
562 specimens (right pot: OSA104247, left pot: OSA185864)

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564 **Fig. 2 a** Locations of the collected *Vincetoxicum pycnostelma* specimens in Kinki and Tokai districts. **b**
565 Location of Kinki and Tokai districts in Japan

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567 **Fig. 3** Results of Bayesian clustering in the STRUCTURE analysis (Pritchard et al. 2010). **a** The
568 proportion of the membership coefficient of 131 individuals in the wild populations at the four sites for
569 each of the inferred clusters for $K = 2$. **b** The proportion of the membership coefficient of 163 individuals
570 in the specimens' seedlings and wild populations at the four sites (right side: specimens' seedlings, left
571 side: wild populations) for each of the inferred clusters for $K = 3$ and $K = 4$. Each column represents an
572 individual

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574 **Fig. 4** Principal coordinates analysis plots of individuals at the four sites (each contains specimens'
575 seedlings and wild populations) based on co-dominant genotypic distances (Smouse and Peakall 1999).
576 Red, green, white and blue symbols represent Sites d, e, g and i, respectively. Axis 1 explains 27.5% of
577 the variance and axis 2 explains 21.4% of the variance

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Figure 1

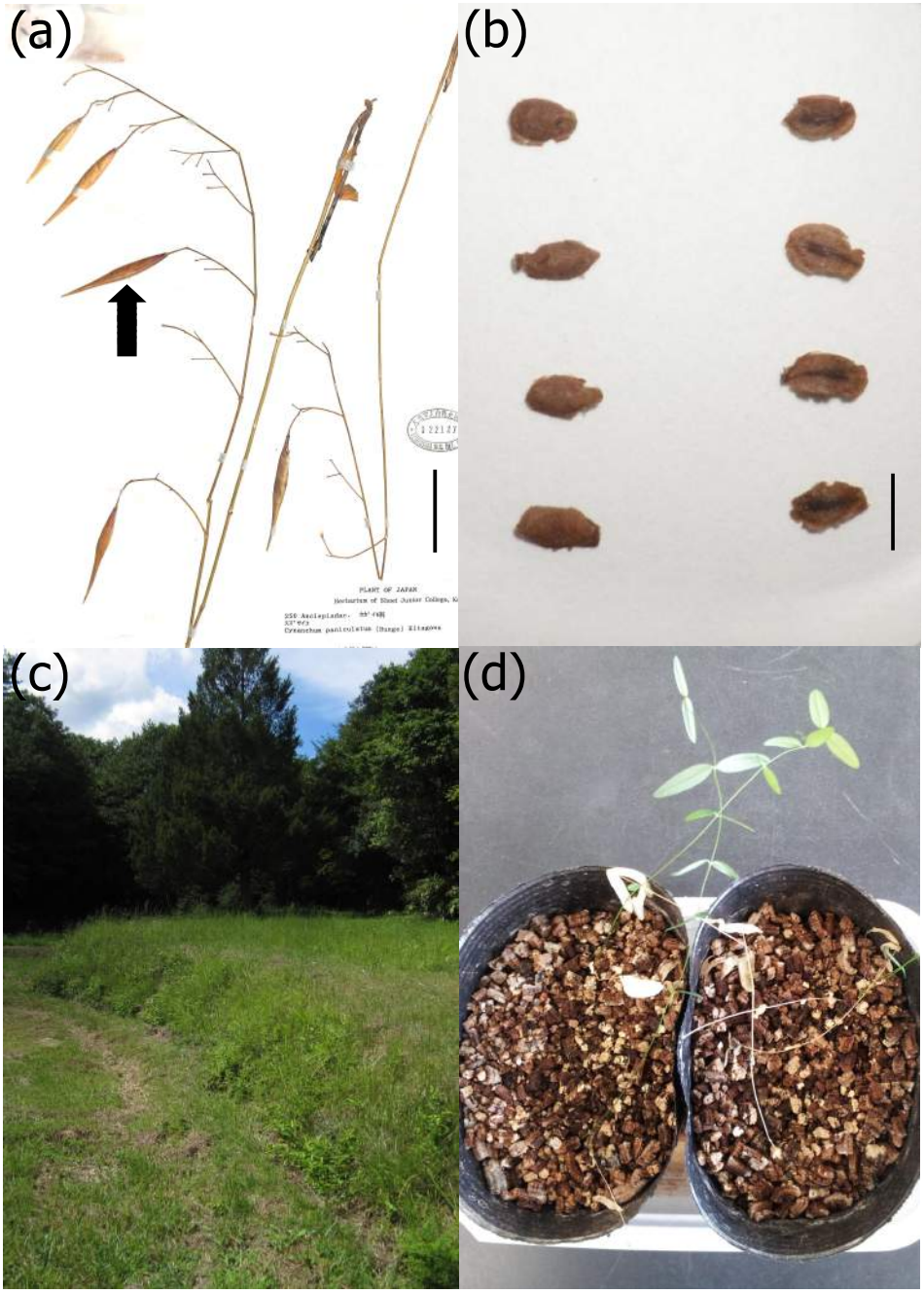


Figure 2

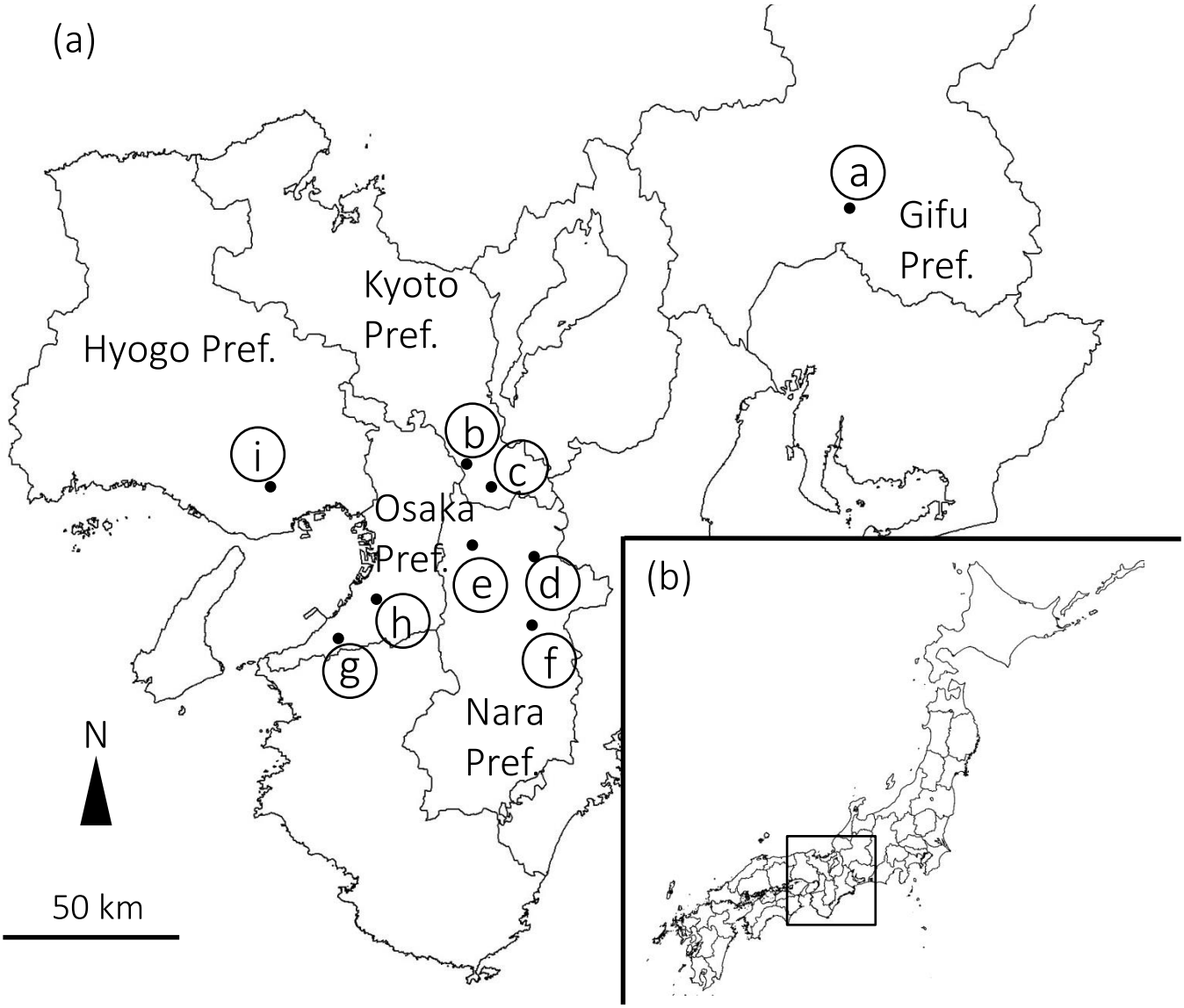


Figure 3

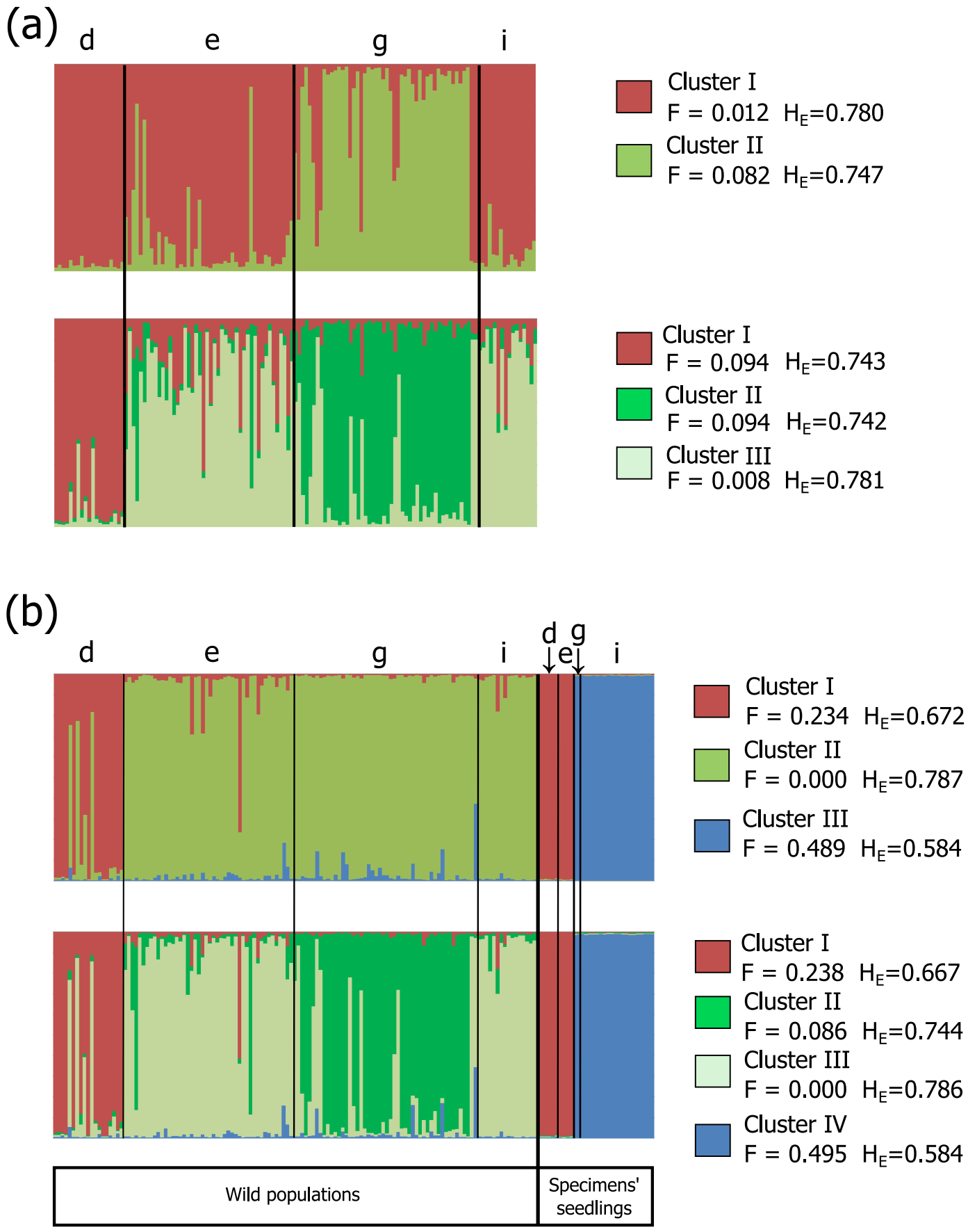


Figure 4

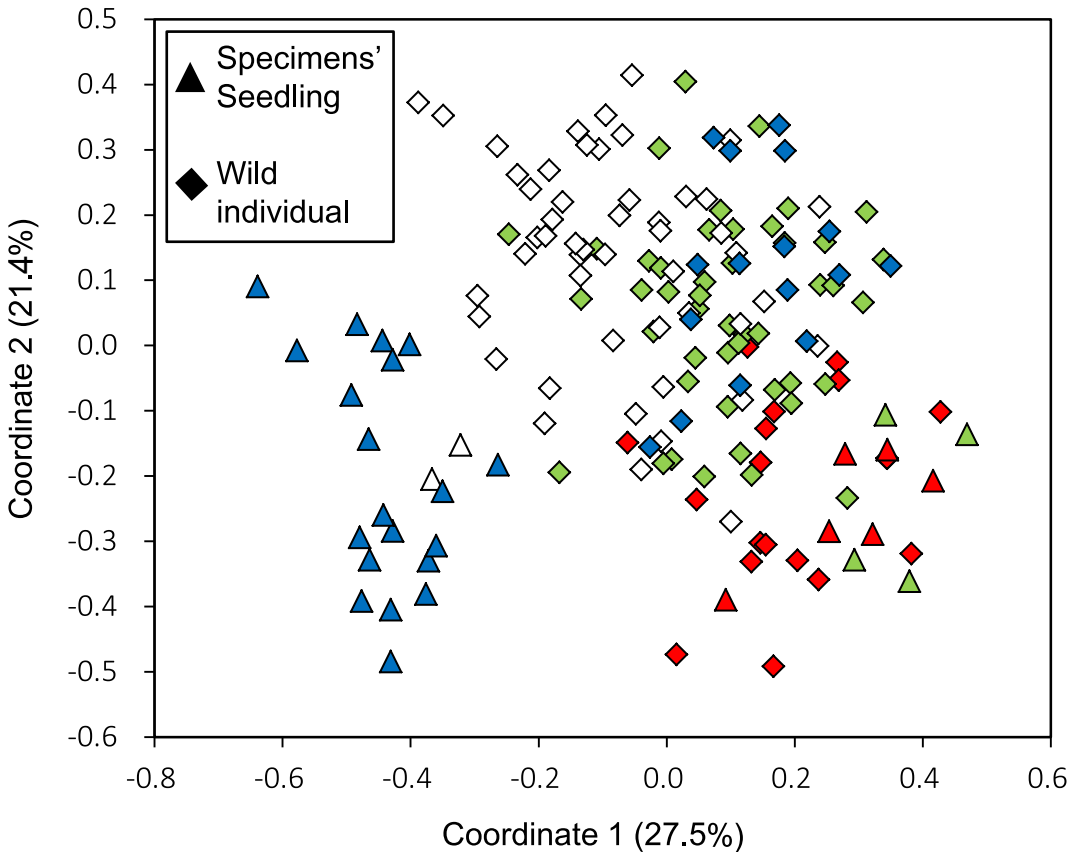
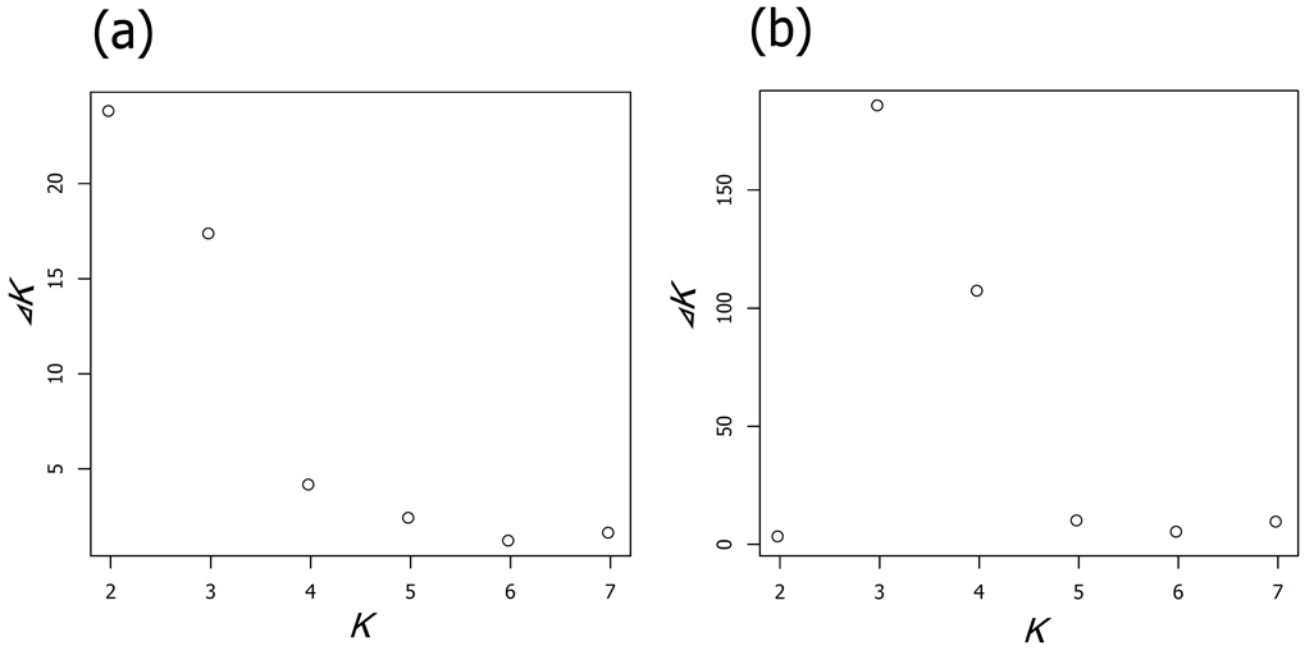


Table S1 Relationships between temperature and number of days in each condition. In the increasing temperature condition, the temperature was alternated between 12 and 25 °C in 24-h intervals after arriving at the final temperature

Increasing temperature condition	Temperature (°C)	4	8	12	16	20	24	28	32	36	12~25
	Number of days	8	5	4	3	2	2	2	2	2	5
Decreasing temperature condition	Temperature (°C)	36	32	28	24	20	16	12	8	4	25
	Number of days	2	2	2	2	2	3	4	5	8	5

Figure S1
The ΔK in the STRUCTRE analysis based on the rate of change in the log probability of data between successive K values (Evanno et al. 2005). (a) The result of 131 individuals in the only wild populations of the four sites. (b) The result of 163 individuals in the specimens' seedlings and wild populations of four sites.



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